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13. ABSTRACT ***(Maximum 200 Words)***

Parkinson's disease (PD) is characterized by a loss of substantia nigra dopaminergic neurons. Here we describe our progress in understanding the role of metabotropic glutamate receptors (mGluRs) as a novel target for the treatment of PD. We have localized mGluR4 in basal ganglia structures, and explored its role in mediating the electrophysiological effects of glutamate in rat brain slices. We have explored the efficacy of mGluR drugs in relieving motor symptoms in hemi-parkinsonian monkeys. We found that group III mGluRs are presynaptic on striatal-pallidal terminals and that they mediate a reduction in IPSC amplitude in the SNr. They also pre-synaptically inhibit EPSCs at the STN-SNr synapse. In this study, we have found that groups I and II mGluRs also have critical roles in regulating basal ganglia function. Group II mGluRs mediate a presynaptic reduction of EPSCs in the SNr and group II agonist LY354740 reverses catalepsy in a rodent model of PD. Pre- and Post-synaptic group I mGluRs are involved in regulation of basal ganglia output nuclei by both excitation and disinhibition. Furthermore, comprehensive characterization of the roles of mGluRs in the basal ganglia raises the possibility that they may provide targets for novel therapeutic agents for treatment of PD.

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor impairments including tremor, rigidity, and bradykinesia. In PD there is a significant loss of nigrostriatal dopamine neurons that results in a series of neurophysiological changes that lead to a pathological excitation of the subthalamic nucleus (STN). The increased activity of STN neurons leads to an increase in glutamate release at STN synapses onto GABAergic projection neurons in the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr). This glutamate-mediated over excitation of the BG output nuclei ultimately leads to a "shutdown" of thalamocortical projections and produces the motor impairments characteristic of PD. Please see Appendix I for a schematic diagram of the normal basal ganglia circuitry compared to the activity changes that occur in PD (Wichmann, 1997). Unfortunately, as the disease progresses, the efficacy of traditional dopamine replacement therapy becomes severely diminished and severe motor and psychiatric side effects can occur (Poewe, 1986). Because of this, much effort has been focused on developing new approaches for the treatment of PD. In these studies we are pursuing a novel therapeutic approach by targeting drugs acting at metabotropic glutamate receptors (mGluRs). Eight mGluR subtypes have been cloned (designated mGluR1-mGluR8) from mammalian brain. Each subtype is classified in one of three classes (I-III). Although the present research focuses on group III mGluRs, in particular mGluR4, during the course of our investigation, we discovered that Groups I and II mGluRs also play crucial roles in regulating BG function. Consequently, we have expanded our mGluR studies to include receptors from the other two groups. During this grant period we focused most of our efforts on specific aims 2 and 3. We characterized the effects of selective mGluR agonists and antagonists on synaptic transmission in the SNr and STN. Finally, we have evaluated the therapeutic potential of mGluR agonists and antagonists in hemi-parkinsonian monkeys.

BODY

Specific Aim I: To localize mGluR4a and 4b receptors in rat and monkey basal ganglia by immunohistochemical techniques using subtype- and isoform- specific antibodies.

We published most of the work proposed for this aim during the first two years of the study (Bradley, 1999). All of the data that we have completed is outlined in our previous reports. Future experiments will employ immunocytochemistry to characterize the receptor distribution of mGluR4a in the basal ganglia of hemi-parkinsonian rhesus monkeys that have been treated with mGluR agonists and antagoinsts. Such experiments will be completed over the remaining course of the study as the monkeys are sacrificed upon the completion of Specific Aim 3. In addition, all of the same characterization and localization experiments will be performed for mGluR4b, the other isoform.

Specific Aim II: To determine the effect of selective group III mGluR agonists and antagonists on synaptic transmission at the major excitatory synapses in the output nuclei of the basal ganglia.

Whole cell patch clamp techniques were used to record the electrophysiological effects of mGluR agonists and antagonists on synaptic transmission in different nuclei of the BG. In particular, the role of mGluRs in the STN and SNr were studied for this grant period.

The STN plays an important role in normal motor function and in PD by providing the major glutamatergic excitatory input to the basal ganglia output nuclei. We find that activation of groups I or III mGluRs, but not group II, causes a depression of excitatory transmission in the STN. In contrast, mGluR activation in the STN has no effect on inhibitory transmission. Further characterization of the group I mediated response revealed that this response is mediated by mGluR1a and not mGluR5. Paired pulse studies suggest that mGluR1 and the group III mGluR-mediated effects are due to a pre-synaptic mechanism. If these receptors are involved in endogenous synaptic transmission in the STN, the data further supports the possibility that

selective drugs targeting mGluRs may provide an alternative approach to the treatment of PD. The corresponding data and further analysis can be found in appendix I. (Awad-Granko, 2001).

The SNr is a primary output nucleus of the basal ganglia. SNr function is controlled by a fine balance between excitatory and inhibitory inputs. The major excitatory input to GABAergic neurons in the SNr arises from glutamatergic neurons in the STN. As previously reported, group I mGluRs are abundant in these nuclei (Marino, 2000) and are likely involved in the modulation of glutamatergic synaptic transmission at excitatory subthalamonigral synapses. To test this, we investigated the effects of group I mGluR activation on excitatory synaptic transmission in putative GABAergic neurons in the SNr. We report that activation of mGluR1 selectively mediates a decrease in synaptic transmission at excitatory synapses. This decrease in excitatory transmission occurs by a presynaptic mechanism, as confirmed by the published electron microscopy data. The corresponding published data and analysis can be found in appendix II. (Wichmann, 2001)

Further studies will complete our characterization of the electrophysiological properties of mGluRs in the BG. Additionally, we intend to publish a complete review of these findings.

Specific Aim III: To evaluate the therapeutic potential of group III agonists in hemi-parkinsonian monkeys.

This aim directly measures the efficacy of mGluR agonists on hemi-parkinsonian monkeys. Primate studies are inherently slow, and these experiments are technically challenging; therefore, despite our expertise and concerted efforts, the progress in this aim is limited compared to progress on the other two aims.

Multiple factors have played a role in the fairly slow progress in these important experiments. A general problem in all of our monkey studies is the current scarcity of suitable Rhesus monkeys. We are currently experiencing wait times of between 2 and 6 months for shipments of animals. The primary reasons why many animals are excluded from this research are concerns about their health; the recent death of a worker at the

Yerkes Regional Primate Center here at Emory has particularly emphasized the potential dangers of the simian Herpes-B agent. This and other potential zoonotic threats are being evaluated during a three-month quarantine period, which each animal has to undergo here at Emory. We feel that only those animals that can be assumed with a reasonable degree of certainty to not carry these simian viral diseases should be used in the experiments, because close contacts between the monkeys and their care-takers cannot be avoided and should be kept as safe as possible. Additional delays arise as consequence of the experimental paradigm itself. As described in previous progress reports, the animals are first behaviorally conditioned and monitored for normal baseline activity levels. They then undergo unilateral intracarotid administrations of the dopaminergic neurotoxin MPTP to induce hemiparkinsonism. One of the time-consuming features of this treatment is that animals tend to recover from partial dopaminergic depletion, so that the treatment has to be repeated several times to achieve a truly stable hemi-parkinsonian state. The recovery may be obvious in as short a period as two weeks after application of the toxin, but in other cases, may take several months to occur. Consequently, the animals have to be observed for extended periods of time (at least three months, in our experience) before recovery can be excluded. The animals treated under this specific aim each had to undergo multiple MPTP injections (four and seven injections, respectively) before a stable hemi-parkinsonian state was accomplished. This has resulted in significant, but unavoidable delays. As the final, usually fairly straightforward step is the preparations for the actual drug administration experiments, the primates have to be equipped with two stainless steel recording chambers to permit chronic access to the motor portions of the basal ganglia. In one of the animals we positioned one of the recording chambers so as to have access to the lateral ventricles as well for intraventricular administration of drugs.

Last year we reported the preliminary finding that the i.c.v. administration of group III agonist R,S- PPG (1 μM) resulted in some antiparkinsonian effects, with an almost two-fold increase of the arm L/R ratio over baseline. This year, we followed up those initial data.

In the current year, we performed a series of experiments with several different drugs and at different doses. Each experiment involves injections and extensive behavioral analysis as was

initially described in the proposal. Thus, we have done considerable work in this past year. The progress includes 6 experiments testing the effects of different doses of R,S- PPG in 2 monkeys. We injected R,S- PPG in the ventricular system at multiple doses. Unfortunately, these results were negative. In fact, 1 μM, 100μM, 1mM, and 10mM produced zero improvement in parkinsonian signs. We have also performed 7 experiments with LY379268. Again, we have not seen any consistent or reproducible effects. An example of the L/R arm movement analysis data is shown in appendix IV.

Because of the negative results with mGluR III agonists, we next decided to do pilot experiments based on our recent progress in aim 2. That is, mGluR 1 inhibited the excitatory transmission in the output nuclei in rats. Hence, MPEP or saline was administered to 1 monkey. MPEP is a very good group I mGluR antagonist that shows subtype-specificity for mGluR5. MPEP was given i.m. at doses of 0.1 mg/kg and 0.5 mg/kg. A vehicle injection of physiological saline showed no effect. The lowest dose of MPEP (0.1 mg/kg) showed no improvement of parkinsonism. The higher dose (0.5 mg/kg) produced disturbing side effects. These side effects included sleepiness, laying down, and hunching over, with zero improvement in parkinsonism. These findings are unexpected and somewhat disappointing given all of the promising electrophysiological and anatomical data that we have reported. Additional studies with different mGluR drugs will be performed as they are made available to us.

KEY RESEARCH ACCOMPLISHMENTS

- **Electrophysiology:**

  1. We demonstrated that activation of groups I or III mGluRs inhibits excitatory transmission in the rat subthalamic nucleus.

  2. We demonstrated that activation of mGluR 1 inhibits glutamatergic transmission in the SNr.

- **Behavior:**

  We found that R,S,-PPG and LY379268, drugs that are specific for the group III mGluRs and MPEP, a drug that is specific for the group I mGluR, mGluR 5 show no anti-parkinsonian effects when administered to hemi-parkinsonian monkeys.

REPORTABLE OUTCOMES

Published Manuscripts

1. Awad-Granko, H. Conn, P.J., Activation of Groups I or III Metabotropic Glutamate Receptors Inhibits Excitatory Transmission in the Rat Subthalamic Nucleus. *Neuropharmacology,* 2001. 41(1): p.32-41.

2. Wittmann, M., Hubert, G.W., Smith, Y., Conn, P.J. Activation of Metabotropic Glutamate Receptor 1 Inhibits Glutamatergic Transmission in the Substantia Nigra Pars Reticulata. *Neuroscience*, 2001. 105(4): p. 881-889.

CONCLUSIONS

This year we have shown that activation of groups I or III mGluRs reduces the excitatory glutamatergic transmission in the STN, and that this reduction is mediated by a presynaptic mechanism. We also show that mGluRs do not play a role in modulating inhibitory transmission in the STN. Additionally, we show that activation of group I mGluRs reduces glutamatergic synaptic transmission in GABAergic SNr neurons and that this effect is also mediated by a presynaptic mechanism. This data advances our understanding of the role that mGluRs play in basal ganglia circuitry, and continues to support the hypothesis that mGluRs may provide a target for the treatment of PD.

The efficacy of these mGluR drugs in an animal model of PD must be determined in order to know for sure whether they are legitimate candidates for the treatment of PD in humans. Our progress in monkey behavioral studies is behind schedule due to the technical and other factors described. However, LY379268 and R,S- PPG, group III mGluR agonists were tested and have not shown any significant anti-parkinsonian effects in monkey studies. MPEP, a mGluR5 antagonist, has been piloted and thus far has not shown any anti-parkinsonian effects. It did produce adverse side effects at 0.5 mg/kg. Continuing studies will be essential to explore the effects of alternative mGluR drugs on the symptoms of PD. Further electrophysiological work will more specifically define the roles of mGluR4 and important related mGluRs in basal ganglia function, and most critically, their value as targets for novel therapeutic drugs in hemi-parkinsonian monkeys.

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APPENDICES

I. Schematic diagram outlining the basal ganglia circuitry in normal and PD brains.

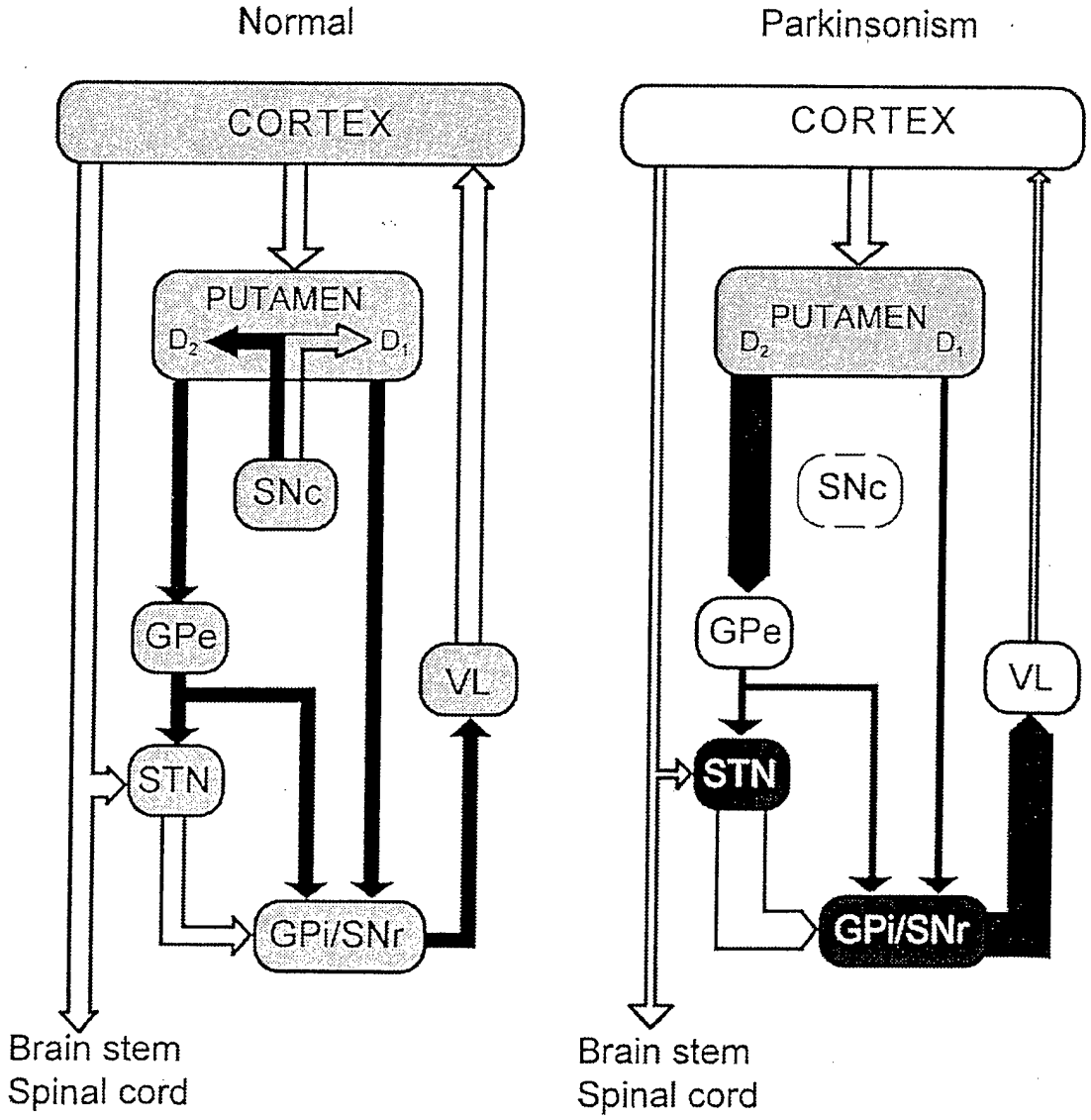
II. Awad-Granko, H., Conn, P.J., Activation of Groups I or III Metabotropic Glutamate Receptors Inhibits Excitatory Transmission in the Rat Subthalamic Nucleus. *Neuropharmacology,* 2001. 41(1): p.32-41.

III. Wittmann, M., Hubert, G.W., Smith, Y., Conn, P.J. Activation of Metabotropic Glutamate Receptor 1 Inhibits Glutamatergic Transmission in the Substantia Nigra Pars Reticulata. *Neuroscience*, 2001. 105(4): p. 881-889.

IV. Data for Specific Aim 3 as mentioned in the Body.

Appendix I

Schematic diagram of the basal ganglia-thalamocortical circuitry under normal conditions. Inhibitory connections are shown as filled arrows, excitatory connections as open arrows. D, direct pathway; I, indirect pathway; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; SNr, substantia nigra, pars reticulata; SNc, substantia nigra, pars compacta; STN, subthalamic nucleus; VL, ventrolateral thalamus.



Activity changes in the basal ganglia-thalamocortical circuitry in Parkinson's disease. Degeneration of the nigrostriatal pathway leads to differential changes in the two striato-pallidal projections, indicated by the thickness of the connecting arrows. Basal ganglia output to the thalamus is increased.

Appendix II

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Activation of groups I or III metabotropic glutamate receptors inhibits excitatory transmission in the rat subthalamic nucleus

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Abstract

The subthalamic nucleus (STN) is a key nucleus in the basal ganglia motor circuit that provides the major glutamatergic excitatory input to the basal ganglia output nuclei. The STN plays an important role in the normal motor function, as well as in pathological conditions such as Parkinson's disease. Development of a complete understanding of the role of the STN in motor control will require a detailed understanding of the mechanisms involved in the regulation of excitatory and inhibitory synaptic transmission in this nucleus. Here, we report that activation of groups I or III metabotropic glutamate (mGlu) receptors, but not group II, causes a depression of excitatory transmission in the STN. In contrast, mGlu receptor activation has no effect on the inhibitory transmission in this nucleus. Further characterization of the group I mGlu receptor-induced effect on EPSCs suggests that this response is mediated by mGlu1 and not mGlu5. Further, paired pulse studies suggest that both the mGlu1 receptor and the group III mGlu receptor-mediated effects are due to a presynaptic mechanism. If these receptors are involved in endogenous synaptic transmission in the STN, these results raise the exciting possibility that selective agents targeting mGlu receptors may provide a novel approach for the treatment of motor disorders involving the STN. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Metabotropic glutamate receptor; mGlu; Subthalamic nucleus; Basal ganglia; Parkinson's disease; Synaptic transmission

1. Introduction

The subthalamic nucleus (STN) is a critical region of the basal ganglia that is involved in the regulation of movement. The STN is the only excitatory glutamatergic nucleus in the basal ganglia motor circuit and provides the major excitatory input to the output nuclei, the substantia nigra pars reticulata and globus pallidus internal capsule. Normal motor function requires an intricate balance between excitation of the output nuclei by glutamatergic neurons from the STN, and inhibition of the output nuclei by GABAergic projections from the striatum (for a review see Wichmann and DeLong, 1997). Because of this, a great deal of effort has been focused on developing a detailed understanding of the circuitry and function of the STN.

Interestingly, recent studies suggest that the major pathophysiological change that occurs in response to loss of nigrostriatal dopamine neurons in Parkinson's disease patients is an increase in activity of STN neurons (Wichmann and DeLong, 1997). The resultant increase in synaptic excitation of GABAergic projection neurons in the output nuclei leads to a 'shutdown' of thalamocortical projections and produces the motor impairment characteristic of Parkinson's disease (DeLong, 1990). Discovery of the pivotal role of increased STN activity in Parkinson's disease has led to a major interest in the development of novel treatment strategies by reducing the neuronal STN activity or STN-induced excitation of basal ganglia output nuclei. Interestingly, surgical lesions (Bergman et al., 1990; Aziz et al., 1991; Guirdi et al., 1996), or inactivation of the STN (Benazzouz et al., 1993; Limousin et al., 1995a,b) are highly effective in the treatment of Parkinson's disease. Development of

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a detailed understanding of the mechanisms involved in the regulation of STN activity could lead to the development of novel therapeutic agents that reduce STN activity without surgical intervention.

Recent studies suggest that metabotropic glutamate (mGlu) receptors play an important role in regulating the activity of neurons in a wide variety of brain regions (for review see Anwyl, 1999; Cartmell and Schoepp, 2000). This has also been demonstrated extensively in basal ganglia structures such as the striatum (Calabresi et al., 1993; Colwell and Levine, 1994; Pisani et al., 1997b), the substantia nigra reticulata (Marino et al., 1999), the substantia nigra pars compacta (Fiorillo and Williams, 1998), the globus pallidus external segment (Maltseva and Conn, 2000), and the STN (Abbott et al., 1997; Awad et al., 2000). If mGlu receptors are involved in regulating synaptic transmission in the STN, this could provide a critical component of regulation of STN activity by glutamatergic afferents from the cortex, pedunculopontine nucleus, and thalamus, or GABAergic afferents from the globus pallidus external segment (Féger et al., 1997). Thus, it will be important to determine whether mGlu receptor activation modulates synaptic transmission in the STN. To date, eight mGlu receptor subtypes have been cloned from mammalian brain and are classified into three major groups based on sequence homologies, second messenger coupling and pharmacological profiles (see Conn and Pin, 1997 for review). Group I mGlu receptors (mGlu1 and mGlu5) couple primarily to Gq, whereas group II (mGlu2 and mGlu3) and group III mGlu receptors (mGlu 4, 6, 7, and 8) couple to Gi/Go. We now report that activation of groups I or III mGlu receptors leads to reduction of excitatory transmission in the STN.

2. Materials and methods

2.1. Materials

L(+)-2-Amino-4-phosphonobutyric acid (L-AP4), (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), (*R,S*)-α-cyclopropyl-4-phosphophenylglycine (CPPG), D(-)-2-amino-5-phosphonopentanoic acid (D-AP-5), 3,5-dihydroxyphenylglycine (DHPG), and methylphenylethynylpyridine (MPEP) were obtained from Tocris Cookson (Ballwin, MO). (+)-2-Aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate (LY354740) was a gift from D. Schoepp (Eli Lilly, Indianapolis, IN). All other materials were obtained from Sigma (St. Louis, MO).

2.2. Tissue preparation

Experiments were performed on STN neurons from 15 to 18 day-old Sprague-Dawley rats. Rats were anesthetized with 7 mg/kg intraperitoneal injection of chloral hydrate prior to decapitation. The brain was removed, mounted and immersed in an oxygenated sucrose–ACSF solution containing: 3 mM KCl, 1.9 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 187 mM sucrose, 20 mM glucose, 26 mM $NaHCO_3$,0.5 mM pyruvate, 0.005 mM glutathione, equilibrated with 95% O_2 and 5% CO_2 at pH 7.4. Parasaggital slices of 300 μm thickness are prepared using a manual VibroSlice (Stoelting, Chicago, IL) and then incubated at room temperature in ACSF containing): 124 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, 20 mM glucose, 26 mM $NaHCO_3$, 0.5 mM pyruvate, 0.005 mM glutathione, equilibrated with 95% O_2 and 5% CO_2 at pH 7.4.

2.3. Electrophysiological recordings

After a 2 h incubation at room temperature, the slices were transferred to a recording chamber mounted on the stage of a Hoffman modulation contrast Olympus microscope and continuously perfused with room temperature oxygenated ACSF (at 1–2 ml/min). Neurons in the STN were visualized using a water-immersion 40× objective. Whole cell patch clamp recordings were made using patch electrodes pulled from borosilicate glass on a Narishige vertical puller. Electrodes were filled with: 140 mM potassium gluconate, 10 mM HEPES, 10 mM NaCl, 0.2 mM EGTA, 2 mM MgATP, 0.2 mM NaGTP. Electrode resistance was 3–5 MΩ. For measurement of synaptically evoked currents, bipolar tungsten electrodes were used to apply stimuli to the internal capsule rostral to the STN at 0.4–12.0 μA every 30–60 s. Synaptically evoked currents were recorded from a holding potential of −60 mV. Slices were bathed in 10 μM bicuculline to block inhibitory transmission for recording EPSCs, and 10 μM D-AP-5 and 20 μM CNQX to block excitatory transmission for recording IPSCs. For paired-pulse facilitation studies, stimuli were given in pairs with intervals ranging from 30 to 50 ms. All drugs were bath applied for 3–5 min in ACSF at 1–2 ml/min. Signals were recorded using a Warner PC-501A patch clamp amplifier (Warner Instrument Corporation, Hamden, CT) and pClamp data acquisition and analysis system (Axon Instruments, Foster City, CA).

2.4. Data analysis

Concentration–response data were fitted with a sigmoidal function using a Marquardt–Levenberg algorithm as implemented in the SigmaPlot program software (SPSS Inc., Chicago, IL). All values are expressed as mean±SEM. Statistical significance was assessed using the Mann–Whitney Rank Sum test.

3. Results

3.1. Groups I or III mGlu receptor activation reduces EPSC amplitude in the STN

A single stimulus to the internal capsule, rostral to the STN induced an inward synaptic current in the presence of 10 μM bicuculline at a holding potential of −60 mV. EPSCs were completely blocked by 20 μM CNQX suggesting that the EPSC is primarily mediated by nonNMDA receptors (data not shown). The group I mGlu receptor-selective agonist, DHPG (100 μM) (Schoepp et al., 1994) caused a transient, reversible depression of EPSCs in STN neurons (Fig. 1A). The group II selective agonist LY354740 (100 nM) (Monn et al., 1997) had no effect on the EPSC amplitude in the STN (Fig. 1B). The group III-selective agonist L-AP4 (1 mM) (Pin and Duvoisin, 1995) also caused a reversible depression of EPSCs in the STN (Fig. 1C). Time courses for each of the group-selective agonist effects on EPSCs are shown (Fig. 1D–F). The DHPG effect is transient and begins desensitizing in the continued presence of the agonist (Fig. 1D). The L-AP4-induced EPSC inhibition showed no apparent desensitization over the period of drug application. However, within approximately 15–20 min of washout of L-AP4, the EPSC returns to within ~80% of predrug amplitude (Fig. 1F).

Concentration–response analysis revealed that both the DHPG and L-AP4 induced inhibition of EPSCs in the STN were concentration-dependent (Fig. 2A and B). The maximal DHPG inhibition was 46.6±3.2% and occurred at 50 μM (Fig. 2A). L-AP4 is more effective at inhibiting EPSCs with a maximal EPSC inhibition of 85.6±1.3% at a concentration of 500 μM (Fig. 2B).

3.2. mGlu receptor activation has no effect on the inhibitory transmission in the STN

Internal capsule stimulation in the presence of 20 μM CNQX and 10 μM D-AP-5 evoked an outward IPSC that was blocked by 10 μM bicuculline (data not shown). DHPG (100 μM) (92.3±23.6% control), LY354740 (100 nM) (98.4±8.8% control) and L-AP4 (1 mM) (99.8±17.6% control) were all without effect on IPSC amplitude in the STN (Fig. 3A–C). Time courses for each of the drug applications are shown indicating no

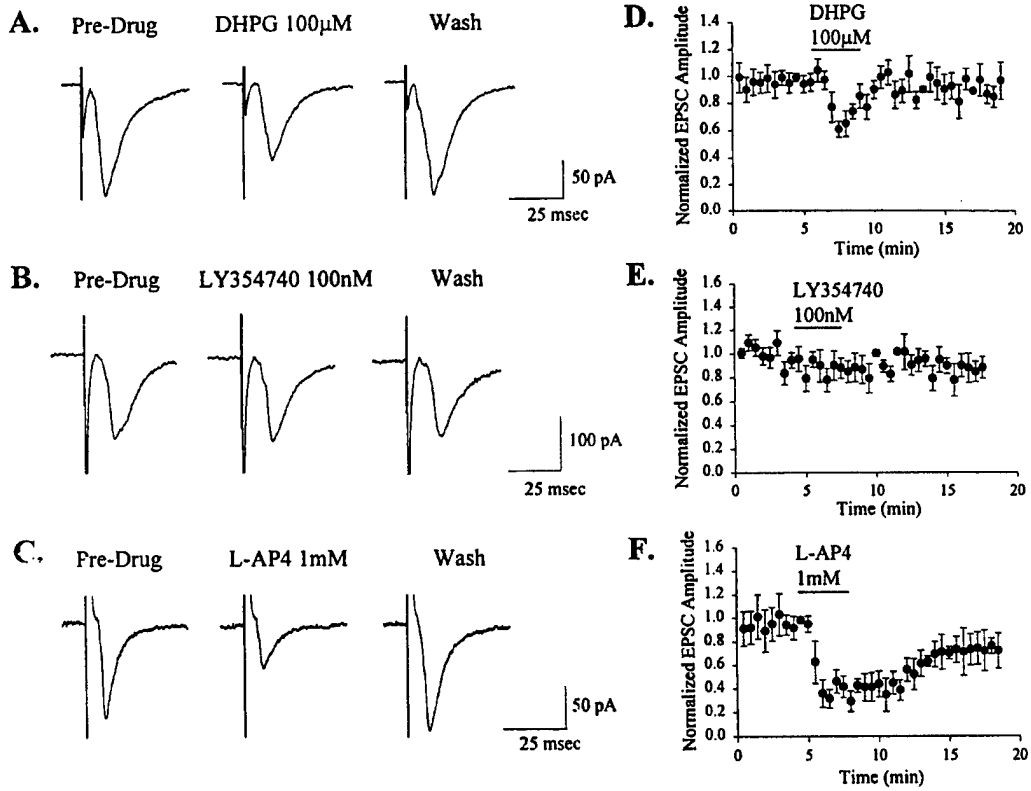

Fig. 1. Activation of groups I or III mGluRs reduces EPSCs in the STN. (A–C) Representative voltage clamp traces of evoked EPSCs in the STN before, during, and after a 3-min application of DHPG (100 μM), LY354740 (100 nM), or L-AP4 (1 mM). (D–F) Average time-course of the effect of each agonist on the normalized EPSC amplitude (agonist application is indicated by a horizontal bar). Each time point represents the mean (±SEM) of data from seven cells for DHPG, five cells for LY354740, and four cells for L-AP4.

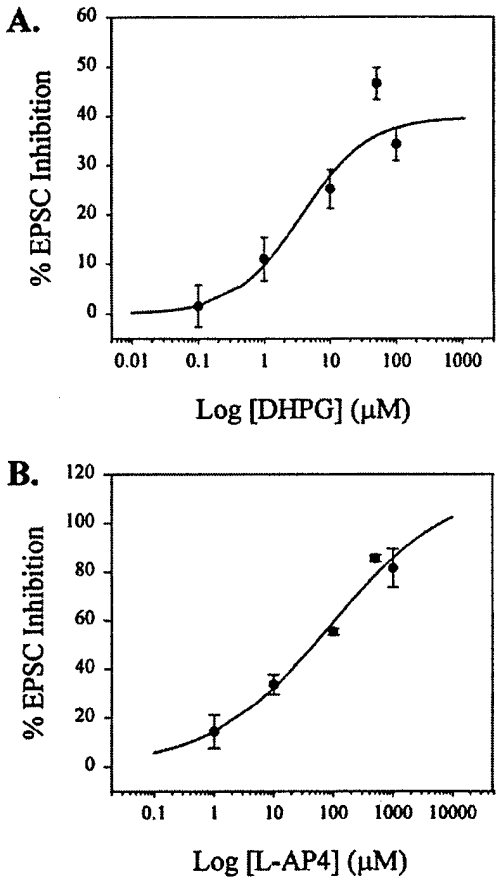

Fig. 2. Concentration–response relationship of groups I and III induced inhibition of EPSCs in the STN. (A) Concentration–response relationship of DHPG-induced inhibition of EPSCs. Maximal DHPG response occurs at a concentration of 50 μM. Each point represents the mean (±SEM) of 3–7 experiments. (B) Concentration–response relationship of L-AP4-induced inhibition of EPSCs. Maximal L-AP4 response occurs at a concentration of 500 μM. Each point represents the mean (±SEM) of 3–4 experiments.

effect of any of the group-selective mGlu receptor agonists on IPSC amplitude (Fig. 3D–F).

3.3. mGlu receptor-induced inhibition of EPSCs in the STN is blocked by selective antagonists

We utilized the newly available pharmacological tools that distinguish between mGlu1 and mGlu5 receptor subtypes to characterize further the group I mediated inhibition of EPSCs in the STN. For these studies, antagonists were bath applied for approximately 10 min prior to the application of the agonist. The mGlu1-selective competitive antagonist LY367385 (300 μM) (Clark et al., 1997; Bruno et al., 1999; Kingston et al., 1999) completely blocked the depression of EPSCs induced by DHPG (10 μM) (3.4±4.88% inhibition, vs 25.2±3.96% inhibition, respectively) (Fig. 4A, B and D). On the other hand, MPEP (10 μM), a noncompetitive antagonist at mGlu5 (Bowes et al., 1999; Gasparini et al., 1999), had no effect on the DHPG-induced inhibition of EPSCs (28.8±10.2% inhibition) (Fig. 4A, C and D). This provides strong evidence that the group I mGlu receptor subtype mediating depression of EPSCs in the STN is mGlu1.

A 10-min preincubation of the group II/III mGlu receptor antagonist CPPG (500 μM) (Toms et al., 1996) blocked the L-AP4-induced inhibition of EPSCs in the STN (31.0±13.8% inhibition, vs 85.6±1.3% inhibition, respectively) (Fig. 5). Since group II mGlu receptors were without effect on the EPSC amplitude in the STN, this provides sufficient evidence that this depression of EPSCs is in fact mediated by group III mGlu receptors.

3.4. The effect of groups I and III selective agonists on EPSCs is mediated by a presynaptic mechanism

To test the hypothesis that group I or III mGlu receptor activation induces a depression of the excitatory synaptic transmission in the STN by a presynaptic mechanism, we determined the effects of group-selective agonists on paired-pulse facilitation of evoked EPSCs. Paired stimulations of the internal capsule were performed at the same stimulus strength with intervals ranging from 30 to 50 ms. Stimulus strength and interpulse intervals were adjusted in each experiment so that the second EPSC was always greater in amplitude than the first (ratio of EPSC2/EPSC1: 1.44±0.117). DHPG (100 μM) reduced the absolute amplitude of the first EPSC, but also increased the ratio of paired-pulse facilitation (ratio of EPSC2/EPSC1: 2.47±0.246) (Fig. 6A and E) significantly. Similarly, L-AP4 (500 μM) also caused a reduction in the EPSC amplitude concomitant with an increase in paired-pulse facilitation (ratio of EPSC2/EPSC1: 3.36±0.463) (Fig. 6B and E). Representative time-courses of the paired pulse facilitation data (Fig. 6C–D) show that the increase in the paired pulse ratio occurs concomitantly with the EPSC inhibition time-courses shown in Fig. 1D and F. In addition, our previously published findings show that neither group I nor group III mGlu receptor activation have any effect on the postsynaptic kainate-evoked current amplitude (Awad et al., 2000). These data combined provide strong evidence in support of the hypothesis that groups I and III mGlu receptors act presynaptically to inhibit the evoked release of glutamate from excitatory glutamatergic terminals in the STN.

4. Discussion

The data presented in this study show that activation of group I or III mGlu receptors reduces the excitatory glutamatergic transmission in the STN, and that this

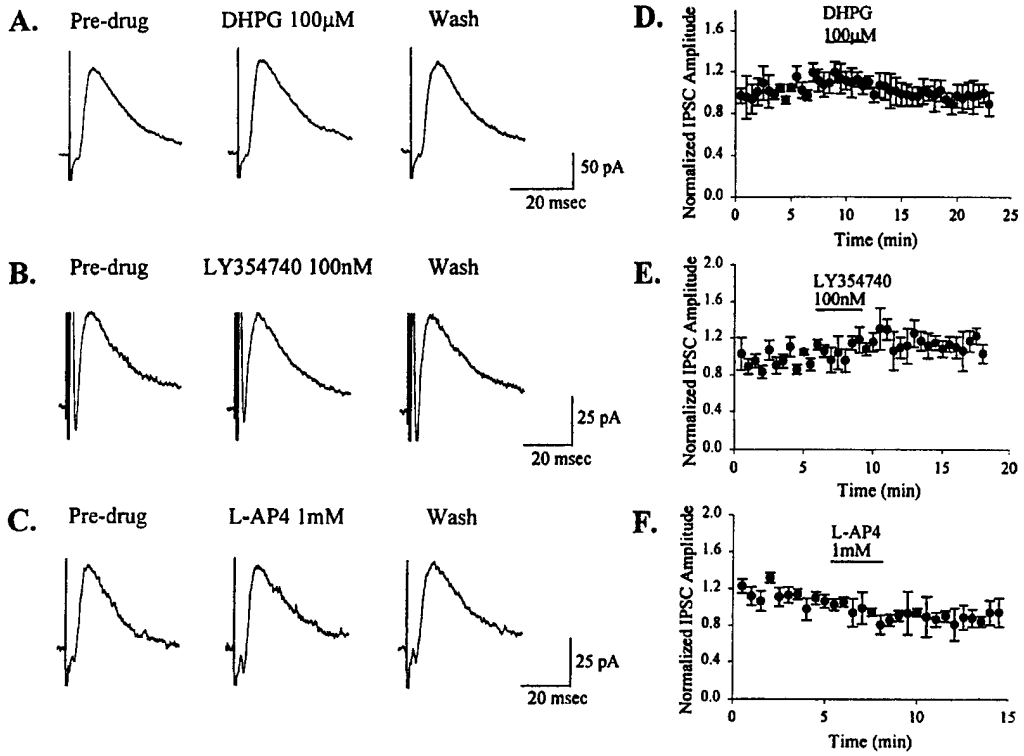

Fig. 3. Activation of groups I, II or III mGluRs has no effect on IPSCs in the STN. (A–C) Representative voltage clamp traces of evoked IPSCs in the STN before, during, and after a 5-min application of DHPG (100 μM), LY354740 (100 nM), or L-AP4 (1 mM). (D–F) Average time-course of the effect of each agonist on the normalized IPSC amplitude (agonist application is indicated by horizontal bar). Each time point represents the mean (±SEM) of data from four cells for DHPG, six cells for LY354740, and three cells for L-AP4.

reduction is likely mediated by a presynaptic mechanism. We also show that mGlu receptors do not play a role in modulating inhibitory transmission in the STN.

Our current findings add to a growing body of literature suggesting that group I mGlu receptors play an important role in regulating basal ganglia function. Group I mGlu receptor mRNA and protein were shown to be expressed throughout the basal ganglia (Testa et al. 1994, 1998; Kerner et al., 1997; Tallaksen-Greene et al., 1998). Both subtypes of group I mGlu receptors are localized postsynaptically in the STN and group I mGlu receptor agonists induce a profound excitation of STN neurons (Abbott et al., 1997; Awad et al., 2000). Group I mGlu receptors, are also heavily localized in the striatum (Shigemoto et al., 1993; Tallaksen-Greene et al., 1998) and substantia nigra pars reticulata (SNr) (Hubert and Smith, 1999) where agonists of these receptors induce excitatory effects (Calabresi et al., 1992; Colwell and Levine, 1994; Pisani et al., 1997b; Marino et al., 1999). In the dopaminergic neurons of the substantia nigra pars compacta (SNc), group I mGlu receptor activation has been shown to elicit a transient hyperpolarization followed by a more pronounced depolarization (Fiorillo and Williams, 1998), and induce a depression of EPSPs (Wigmore and Lacey, 1998). Behavioral studies combined with studies of changes in 2-deoxyglucose and fos immunoreactivity suggest that injection of group I mGlu receptor agonists into the striatum or the STN induces rotational behavior (Sacaan et al. 1991, 1992; Kaatz and Albin, 1995; Feeley Kearney et al., 1997). Taken together, these data suggest that group I mGlu receptors function at multiple levels of the basal ganglia circuit to regulate activity of neurons in the STN and output nuclei.

While group I mGlu receptor agonists induce a number of physiological responses in the basal ganglia, recent studies with the newly developed mGlu1 receptor and mGlu5 receptor ligands suggest that the physiological roles of the group I mGlu receptor subtypes are highly segregated. In the SNr, both mGlu1 and mGlu5 receptor subtypes are localized postsynaptically (Hubert and Smith, 1999). However, mGlu1 mediates the physiological effects of group I mGlu receptor activation in this nucleus (Marino et al., 1999). On the other hand, the converse is true in the STN where both mGlu1 and mGlu5 receptor subtypes are postsynaptically localized, but all physiological effects of group I mGlu receptor activation are mediated by mGlu5 (Awad et al., 2000). Further, our studies indicate that group I mGlu receptor activation in the SNr causes an inhibition of IPSCs

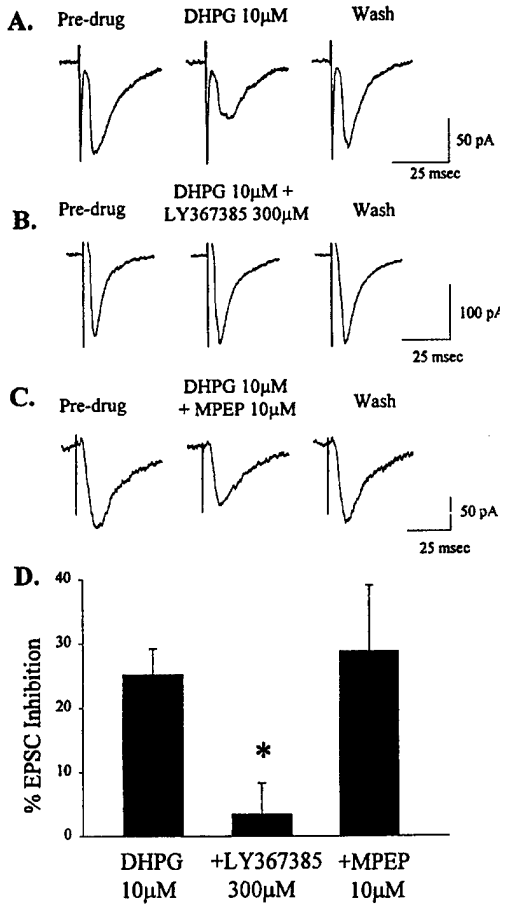

Fig. 4. The group I-induced inhibition of EPSCs is mediated by mGluR1. (A) Representative voltage clamp EPSC traces before, during, and after application of DHPG (10 μM). (B) Effect of a 10-min preapplication of the mGluR1-selective antagonist LY367385 (300 μM) on the DHPG-induced inhibition of EPSCs. (C) Effect of a 10 minute preapplication of the mGluR5-selective antagonist MPEP (10 μM) on the DHPG-induced inhibition of EPSCs. (D) Mean data±SEM of % EPSC inhibition by DHPG (10 μM) in the absence and presence of selective antagonists. Each bar represents average data from four cells (*$p<0.05$, Mann–Whitney test).

(Wittmann et al., 2000) which is believed to be mediated by both mGlu1 and mGlu5 receptor subtypes (unpublished observations).

The present finding that mGlu1 regulates excitatory synaptic transmission in the STN is especially interesting in light of the fact that group I mGlu receptors have been traditionally viewed as primarily postsynaptic receptors that play roles in modulation of postsynaptic cell excitability. Increasing evidence suggests that group I mGlu receptors may also play important roles in the modulation of synaptic transmission at both excitatory and inhibitory synapses throughout the brain (Calabresi et al., 1992; Gereau and Conn, 1995; Bonci et al., 1997; Manzoni et al., 1997; Anwyl, 1999; Cartmell and Schoepp, 2000). Our finding that mGlu1 activation inhibits glutamate release in the STN is consistent with our previous finding of slight mGlu1a immunoreactivity on presynaptic terminals at asymmetric synapses in the STN (Awad et al., 2000). This reduction in excitatory transmission is interesting because it has the opposite effect on the activity of STN neurons than that seen postsynaptically by activation of mGlu5. This could provide a mechanism for differential modulation of STN neuron activity by selectively targeting either mGlu1 or mGlu5. It will be important to determine the net effect of group I mGlu receptor activation in light of these opposing effects in different basal ganglia regions.

Our finding that group III mGlu receptor activation leads to an inhibition of synaptic excitation in the STN is consistent with both the anatomical and physiological studies in other basal ganglia regions. Anatomical studies have demonstrated the presynaptic localization of the mGlu7 receptor subtype at corticostriatal terminals as well as striatopallidal terminals and striatonigral terminals (Kosinski et al., 1999). In addition, presynaptic localization of mGlu4 has been demonstrated at striatopallidal terminals (Bradley et al., 1999b). Group III mGlu receptors were shown to reduce excitatory transmission at the corticostriatal synapse (Pisani et al., 1997a) and at excitatory synapses in the SNc (Wigmore and Lacey, 1998). Group III mGlu receptors have also been shown to reduce both excitatory and inhibitory transmissions in the SNr (Wittmann et al., 2000). Unfortunately, drugs are not available to allow a clear determination of the specific group III mGlu receptor subtypes that mediate these effects. However, previous immunocytochemical studies reveal that mGlu4 and mGlu7 receptors are present in the STN (Bradley et al., 1999a,b; Kosinski et al., 1999), whereas mGlu6 and mGlu8 are not. Thus, the most likely candidates for group III mGlu receptors mediating this effect in the STN are mGlu4 and mGlu7.

Interestingly, group II mGlu receptor activation has no effect on the excitatory transmission in the STN. This is consistent with the previous reports indicating little or no mGlu2/3 immuno-reactivity in the STN (Testa et al., 1998). However, this is interesting in light of increasing evidence demonstrating the role of group II mGlu receptors in the modulation of transmission in several basal ganglia regions. Group II mGlu receptors are localized presynaptically at asymmetric synpases in the SNr and inhibit excitatory synaptic transmission at the STN–SNr synapse (Bradley et al., 2000). Group II mGlu receptors have also been shown to be expressed in the striatum and the SNc (Testa et al., 1998) and they reduce excitatory transmission in these nuclei (Lovinger and McCool, 1995; Wigmore and Lacey, 1998).

The major sources of glutamatergic afferents into the STN arise from the cortex, thalamus, and pedunculopontine nucleus (PPN) (Parent and Hazrati, 1995; Féger et al., 1997). Corticosubthalamic terminals were shown to

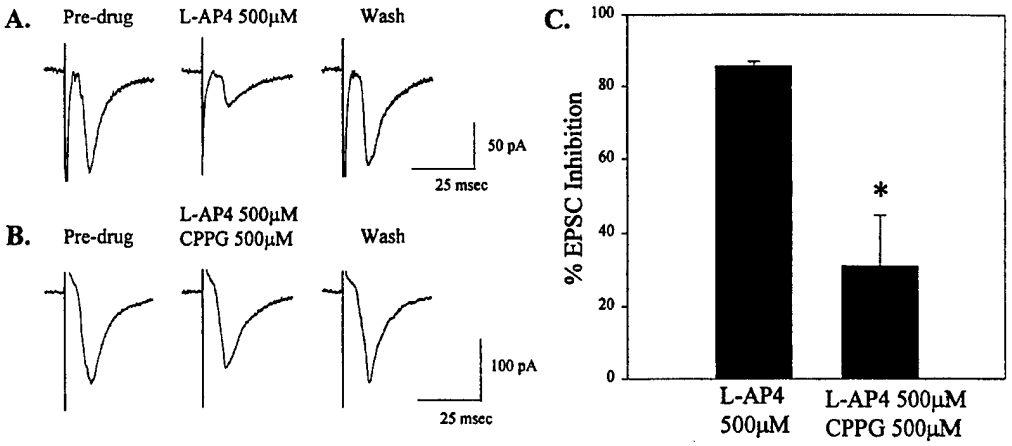

Fig. 5. The group III-induced inhibition of EPSCs is attenuated by a groups II/III antagonist. (A) Representative voltage clamp EPSC traces before, during, and after application of L-AP4 (500 μM). (B) Effect of a 10-min preapplication of the groups II/III mGluR antagonist CPPG (500 μM) on the L-AP4-induced inhibition of EPSCs. (C) Mean data±SEM of % EPSC inhibition by L-AP4 (500 μM) in the absence and presence of CPPG. Each bar represents average data from three cells (*$p<0.05$, Mann–Whitney test).

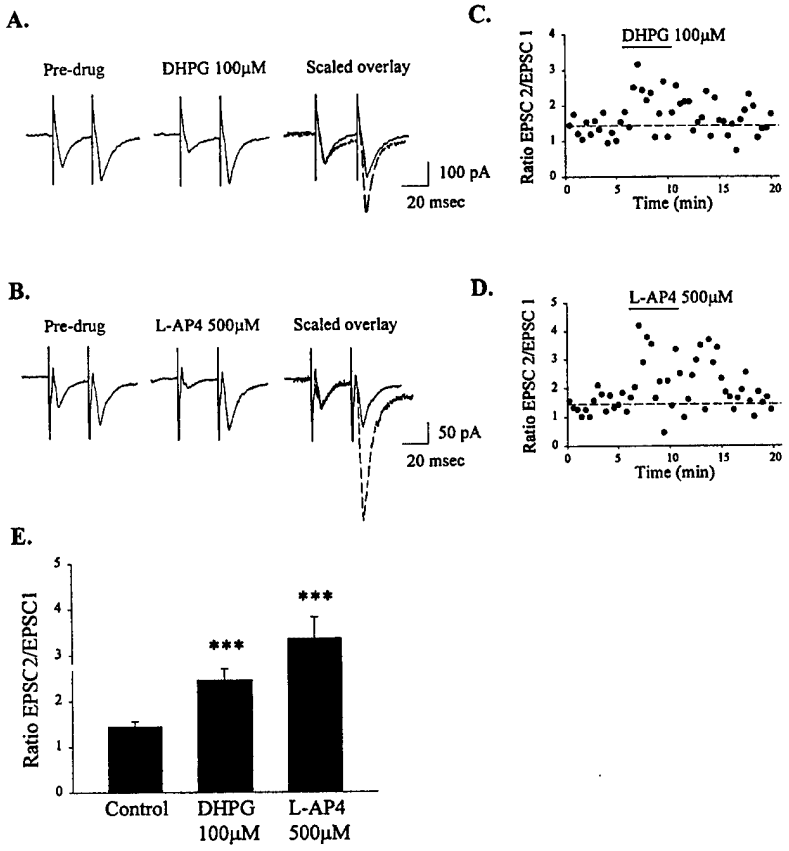

Fig. 6. Inhibition of EPSCs by activation of groups I and III mGluRs is mediated by a presynaptic mechanism. (A–B) Representative voltage clamp traces of paired evoked EPSCs before and during application of DHPG (100 μM) or L-AP4 (500 μM). Scaled overlay represents the superimposed traces of the predrug condition (solid line) and the agonist condition (dashed line; trace scaled to the first EPSC of control condition). (C–D) Representative time-courses of the paired pulse ratio data in one experiment each for DHPG (100 μM) and L-AP4 (500 μM). The dashed line indicates the average predrug ratio. (E) Mean data±SEM of the ratio of the second EPSC to the first in control (n=13), DHPG (n=8) and L-AP4 (n=5) conditions (***$p<0.005$, Mann–Whitney test).

be highly enriched with glutamate immunoreactivity (Bevan et al., 1995). Interestingly, the same postsynaptic structures in the STN that receive this cortical input also receive synaptic afferents from GABAergic pallidal-like terminals, suggesting that both glutamatergic and GABAergic afferents converge onto a single STN cell (Bevan et al., 1995). If this is the case, then it is likely that selective modulation of excitatory transmission in the STN with group I or III mGlu receptor agonists could lead to a reduction in the STN activity without affecting the inhibitory transmission. Electrical stimulation of either the PPN or the parafasicular thalamic nucleus (PF) have also been shown to induce an excitatory glutamatergic response in STN neurons (Hammond et al., 1983; Mouroux and Feger, 1993). Pharmacological stimulation of the PF with carbachol results in an increase in the discharge rate of STN neurons (Mouroux et al., 1995). However, in the present study it is important to note that we are unable to determine the source of the afferents being modulated by mGlu receptors. It is likely that by stimulating the internal capsule, we are stimulating afferents from multiple sources. Further studies are necessary to determine the sources of these afferents, and whether or not different afferents have differential mGlu receptor expression and show differential mGlu receptor actions.

Taken together, these studies demonstrate that groups I and III mGlu receptors are involved in the reduction of excitatory transmission in the STN. These receptors may provide exciting new targets for the development of pharmacological treatments of disorders caused by an alteration in the activity of the STN, such as Parkinson's disease, Huntington's disease, and Tourette's syndrome. By selectively targeting different mGlu receptor subtypes with specific mGlu receptor agonists or antagonists it may be possible to restore the balance necessary for normal basal ganglia function. However, it is important to note that the use of antagonists of mGlu receptors to treat these various disorders will depend upon the physiological activation of these receptors by endogenous glutamate, which is yet to be demonstrated. In addition, owing to the differential effects of mGlu receptor activation in the different regions of the basal ganglia, it will be important to determine the overall net effect of activation or inhibition of mGlu receptors in the whole animal prior to the consideration of agents modulating these receptors for therapeutic use.

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Appendix III

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ACTIVATION OF METABOTROPIC GLUTAMATE RECEPTOR 1 INHIBITS GLUTAMATERGIC TRANSMISSION IN THE SUBSTANTIA NIGRA PARS RETICULATA

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Abstract—The substantia nigra pars reticulata is a primary output nucleus of the basal ganglia motor circuit and is controlled by a fine balance between excitatory and inhibitory inputs. The major excitatory input to GABAergic neurons in the substantia nigra arises from glutamatergic neurons in the subthalamic nucleus, whereas inhibitory inputs arise mainly from the striatum and the globus pallidus. Anatomical studies revealed that metabotropic glutamate receptors (mGluRs) are highly expressed throughout the basal ganglia. Interestingly, mRNA for group I mGluRs are abundant in neurons of the subthalamic nucleus and the substantia nigra pars reticulata. Thus, it is possible that group I mGluRs play a role in the modulation of glutamatergic synaptic transmission at excitatory subthalamonigral synapses. To test this hypothesis, we investigated the effects of group I mGluR activation on excitatory synaptic transmission in putative GABAergic neurons in the substantia nigra pars reticulata using the whole cell patch clamp recording approach in slices of rat midbrain. We report that activation of group I mGluRs by the selective agonist (*R,S*)-3,5-dihydroxyphenylglycine (100 μM) decreases synaptic transmission at excitatory synapses in the substantia nigra pars reticulata. This effect is selectively mediated by presynaptic activation of the group I mGluR subtype, mGluR1. Consistent with these data, electron microscopic immunocytochemical studies demonstrate the localization of mGluR1a at presynaptic sites in the rat substantia nigra pars reticulata.

From this finding that group I mGluRs modulate the major excitatory inputs to GABAergic neurons in the substantia nigra pars reticulata we suggest that these receptors may play an important role in basal ganglia functions. Studying this effect, therefore, provides new insights into the modulatory role of glutamate in basal ganglia output nuclei in physiological and pathophysiological conditions.

Key words: basal ganglia, DHPG, movement disorders, presynaptic, subthalamic nucleus.

The basal ganglia is a highly interconnected group of subcortical nuclei in the vertebrate brain that plays a critical role in the control of movements. The GABA-containing projection neurons of the substantia nigra pars reticulata (SNr) together with those of the entopeduncular nucleus (EPN) are the main output nuclei of the basal ganglia (Grofova et al., 1982). GABAergic projection neurons in the SNr receive inputs from the striatum, the primary input nucleus of the basal ganglia, via two parallel but opposing pathways (DeLong, 1990; Bergman et al., 1990). The 'direct pathway' originates from a subpopulation of GABAergic striatal neurons that project directly to the SNr and, thereby, inhibit activity of these output neurons. The 'indirect pathway' originates from a different population of GABAergic striatal neurons that project to the SNr via the external segment of the globus pallidus (or globus pallidus in rats) and the subthalamic nucleus (STN). In turn, the STN provides excitatory glutamatergic inputs to the SNr. An intricate balance of activity between these pathways is believed to be necessary for a normal fine tuning of motor function, and the disruption of this balance leads to various movement disorders (Wichmann and DeLong, 1997, 1998). In Parkinson's disease (PD), the loss of nigrostriatal dopamine neurons results in a decreased activity of the direct pathway and an increased activity of the indirect pathway which leads to an increased firing

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Abbreviations: ACSF, artificial cerebrospinal fluid; DHPG, (*R,S*)-3,5-dihydroxyphenylglycine; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EPN, entopeduncular nucleus; EPSC, excitatory postsynaptic current; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); LY367385, (*S*)-(+)-α-amino-4-carboxy-2-methylbenzoacetic acid; mGluR, metabotropic glutamate receptor; MPEP, methyl-phenyl-ethynylpyridine; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus.

of STN neurons (Wichmann and DeLong, 1997). The overactive glutamatergic excitation of GABAergic neurons in the output nuclei of the basal ganglia (EPN/SNr) by the STN is believed to underlie the motor symptoms of PD (DeLong, 1990).

Glutamate is the major excitatory transmitter in the mammalian brain. While much effort has been focused on studying fast glutamatergic synaptic transmission via α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and *N*-methyl-D-aspartate (NMDA) receptors, recent studies indicate that glutamate also has important modulatory influences on excitatory synaptic transmission by action on metabotropic glutamate receptors (mGluRs).

These G protein-coupled receptors are highly expressed throughout the basal ganglia (Testa et al., 1994, 1998; Kerner et al., 1997; Kosinski et al., 1998, 1999; Bradley et al., 1999a,b). To date, eight mGluR subtypes (mGluR1–8) have been cloned, and are classified into three major groups based on sequence homology, coupling to second messenger systems, and selectivities for various agonists (Conn and Pin, 1997). Group I mGluRs (mGluR1 and 5) couple to G_q and activate phosphoinositide hydrolysis, while group II mGluRs (mGluR2 and 3) and group III mGluRs (mGluR4, 6, 7 and 8) couple to $G_{i/o}$ and associated effector systems such as adenylyl cyclase. The mGluRs (with the exception of mGluR6) are widely distributed throughout the CNS and play important roles in regulating cell excitability and synaptic transmission at excitatory and inhibitory synapses (for review see Conn and Pin, 1997).

Previous anatomical studies have shown that both group I mGluR subtypes (mGluR1 and mGluR5) are present in STN and SNr neurons (Testa et al., 1994). Furthermore, recent studies in our laboratory have shown that activation of postsynaptically localized group I mGluRs produces a robust direct depolarization of putative projection neurons in both the SNr (Marino et al., 1999) and STN (Awad et al., 2000) in rats. Thus, group I mGluRs could play an important role in increasing the net excitatory drive onto SNr neurons and, thereby, increase the overall basal ganglia output. However, in addition to these postsynaptic effects, group I mGluRs in the hippocampus have also been shown to reduce excitatory and inhibitory synaptic transmission (Gereau and Conn, 1995; Manzoni and Bockaert, 1995). If group I mGluRs have similar effects in the SNr, this could influence the overall impact of group I mGluR activation on transmission at STN–SNr synapses. To investigate this issue, we performed a series of *in vitro* whole cell patch clamp recording studies to determine whether activation of group I mGluRs modulates excitatory glutamatergic transmission in SNr neurons.

EXPERIMENTAL PROCEDURES

Materials

[*R*-(*R**,*S**)]-6-(5,6,7,8-Tetrahydro-6-methyl-1,3-dioxolo[4,5-*g*]-isoquinolin-5-yl)furo[3,4-*e*]-1,3-benzodioxol-8(6*H*)-one (bicuculline), 7-hydroxyiminocyclopropan-[*b*]chromen-1a-carboxylic acid ethyl ester, (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG), (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) and methyl-phenyl-ethynylpyridine (MPEP) were obtained from Tocris (Ballwin, MO, USA). All other materials were obtained from Sigma (St. Louis, MO, USA).

Electrophysiology

Whole cell patch clamp recordings were obtained under visual control as previously described (Marino et al., 1998; Bradley et al., 2000). Fifteen- to eighteen-day-old Sprague–Dawley rats (Charles River, USA) were used for all patch clamp studies. The animals were anesthetized with chloral hydrate (700 mg/kg) and transcardially perfused with an ice cold sucrose buffer (in mM: sucrose, 187; KCl, 3; $MgSO_4$, 1.9; KH_2PO_4, 1.2; glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2). Brains were rapidly removed and submerged in ice cold sucrose buffer. Parasagittal slices (300 μm thick) were made using a Vibraslicer (WPI). Slices were transferred to a holding chamber containing normal artificial cerebrospinal fluid (ACSF; in mM: NaCl, 124; KCl, 2.5; $MgSO_4$, 1.3; NaH_2PO_4, 1.0; $CaCl_2$, 2.0, glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2). In all experiments, 5 μM glutathione and 500 μM pyruvate were included in the sucrose buffer and holding chamber. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continually perfused with room temperature ACSF (~3 ml/min, 23–24°C). Neurons in the SNr were visualized with a 40× water immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige vertical patch pipette puller and filled with a mixture of (in mM) potassium gluconate, 140; HEPES, 10; NaCl, 10; EGTA, 0.6; NaGTP, 0.2; MgATP, 2; pH adjusted to 7.4 with 0.5 N KOH. Electrode resistance was 3–7 MΩ.

GABAergic SNr neurons were identified according to previously established electrophysiological criteria (Richards et al., 1997). GABAergic neurons exhibit spontaneous repetitive firing, short duration action potentials, little spike frequency adaptation, and a lack of inward rectification, whereas dopaminergic neurons display no or low frequency spontaneous firing, longer duration action potentials, strong spike frequency adaptation, and a pronounced inward rectification. All data presented in this study are from neurons which fit the electrophysiological criteria of GABAergic neurons.

Excitatory postsynaptic currents (EPSCs) were evoked with bipolar tungsten electrodes (0.4–1.2 μA every 30 s). The stimulation electrode was placed into the STN or the cerebral peduncle rostral to and outside the SNr. EPSCs were recorded from a holding potential of −60 mV. Bicuculline (10 μM) was bath applied during all EPSC recordings to block inhibitory transmission.

Values are expressed as mean ± S.E.M. Statistical significance was assessed using Student's *t*-test. $P < 0.05$ was set as the limit of statistical significance.

Immunohistochemical method

Animal perfusion and preparation of tissue. Three male Sprague–Dawley rats, 14 days old, were deeply anesthetized with chloral hydrate (400 mg/kg) and transcardially perfused with cold, oxygenated Ringer's solution followed by 100 ml of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) and 50 ml of cold PB. Next, the brain was removed from the skull and stored in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) before being sliced on a vibrating microtome into 60-μm transverse sections. These sections were then treated with 1.0% sodium borohydride for 20 min and rinsed in PBS.

Electron microscope immunohistochemistry. For electron microscopy studies, the sections were treated with cryoprotectant for 20 min and transferred to a −80°C freezer for an additional 20 min, returned to a decreasing gradient of cryoprotectant solutions and rinsed in PBS. Sections then underwent immunocytochemical procedures for the immunoperoxidase

localization of mGluR1a. The sections were preincubated at room temperature in a solution containing 10% normal horse serum and 1.0% bovine serum albumin in PBS for 1 h. They were then incubated for 48 h at 4°C in a solution containing a monoclonal antibody raised against the C-terminus of mGluR1a (Pharmingen, San Diego, CA, USA) diluted at 0.25 μg/ml in the preincubation solution. This antibody has been shown to be highly specific for mGluR1a in both immunoblot and immunocytochemical studies performed on rat tissue and transfected HEK cells. Furthermore, this staining is blocked by preadsorption with the antigenic peptide (Shigemoto et al., 1994; Petralia et al., 1997). Next, the sections were rinsed in PBS and transferred for 1 h at room temperature to a secondary antibody solution containing biotinylated horse anti-mouse IgGs (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in the primary antibody diluent solution. After rinsing, sections were put in a solution containing 1:100 avidin–biotin–peroxidase complex (Vector). The tissue was then washed in PBS and 0.05 M Tris buffer before being transferred to a solution containing 0.01 M imidazole, 0.0005% hydrogen peroxide, and 0.025% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) in Tris for 7–10 min. The sections were then transferred to PB (0.1 M, pH 7.4) for 10 min and exposed to 1% osmium tetroxide for 20 min. Afterwards, they were rinsed with PB and dehydrated in an increasing gradient of ethanol. Uranyl acetate (1%) was added to the 70% alcohol to increase contrast at the electron microscope. The sections were then treated with propylene oxide before being embedded in epoxy resin (Durcupan, ACM, Fluka, Buchs, Switzerland) for 12 h, mounted on microscope slides and placed in a 60°C oven for 48 h.

One block of SNr tissue was taken from each rat and glued on the top of resin blocks with cyanoacrylate glue. They were cut into 60-nm ultrathin sections with an ultramicrotome (Ultracut T2, Leica, Nussloch, Germany) and serially collected on single-slot Pioloform-coated copper grids. The sections were stained with lead citrate for 5 min and examined with a Zeiss EM-10C electron microscope (Thornwood, NY, USA). Electron micrographs were taken at low and high magnifications to characterize the nature of mGluR1a-immunoreactive elements in the SNr.

The anesthesia and euthanasia procedures were carried out according to the National Institutes of Health Guidelines and have been accepted by the Institutional Animal Care and Use Committee of Emory University. All efforts were made to reduce the number of animals used and to minimize animal suffering.

RESULTS

Whole cell patch clamp experiments were performed at electrophysiologically identified GABAergic neurons of the SNr in midbrain slices. EPSCs were elicited by stimulation of the STN or the cerebral peduncle, rostral to the SNr with bipolar stimulation electrodes. We have previously demonstrated that EPSCs elicited by STN stimulation with this protocol are of a constant latency and blocked by 6-cyano-7-nitroquinoxaline-2,3-dione

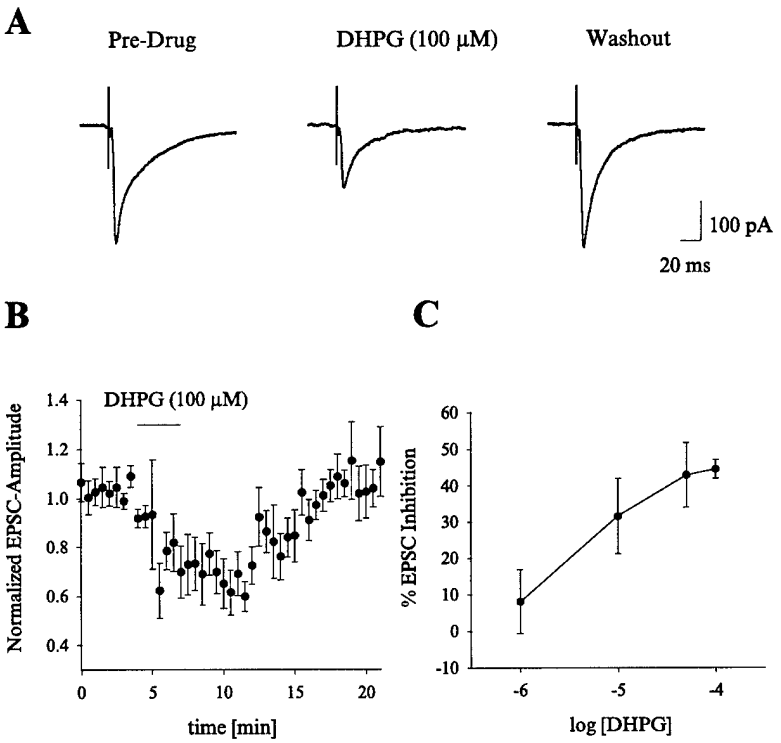

Fig. 1. Application of DHPG suppresses EPSCs in SNr GABAergic neurons. (A) Example traces of evoked EPSCs before (Pre-Drug), during (DHPG) and after (Washout) brief bath application of 100 μM DHPG. (B) Average time course of the effect of 100 μM DHPG demonstrating that the effect of DHPG is reversible. Values were normalized to a Pre-Drug baseline. Each point represents the mean (±S.E.M.) of data from six cells. (C) Dose–response relationship of the effect of DHPG on EPSC amplitudes showing that the effect of DHPG is dose-dependent with an EC_{50} of about 5 μM. Each point represents the mean (±S.E.M.) of data from four to six cells.

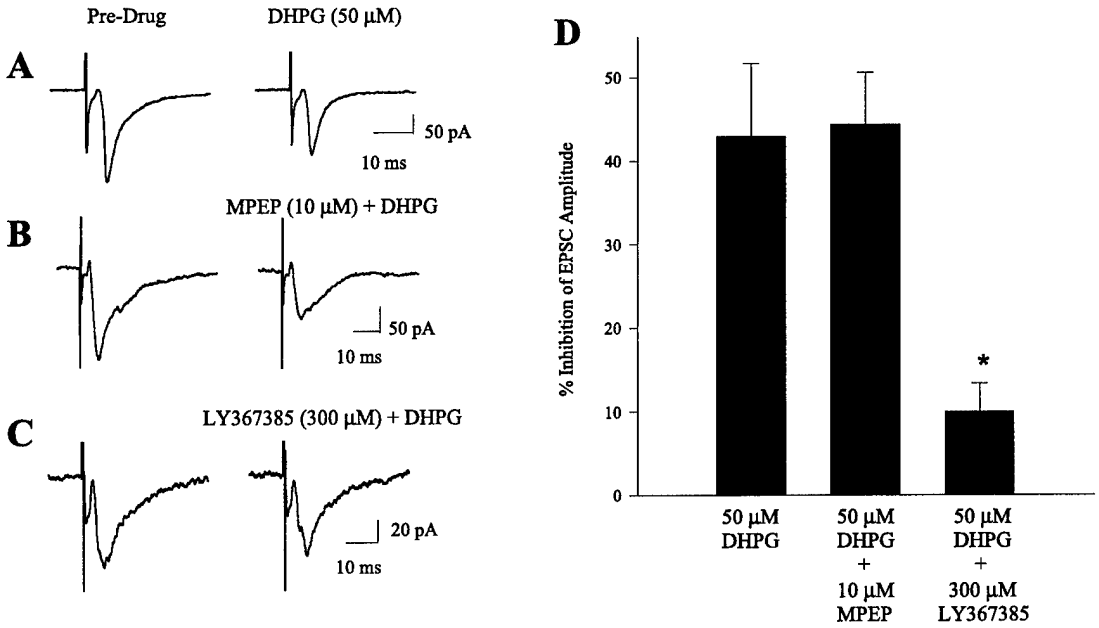

Fig. 2. The effect of DHPG on EPSCs is mediated by mGluR1 but not mGluR5. (A–C) Example traces showing the effects of mGluR1 and mGluR5 selective antagonists on the 50 μM DHPG-induced effects on EPSCs. Antagonists include the mGluR5 selective, non-competitive antagonist MPEP 10 μM (B) and the mGluR1 selective, competitive antagonist LY367385 300 μM (C). (D) Bar graph showing the average effect of mGluR1 and mGluR5 selective antagonists on the DHPG-induced effect. Each bar represents the mean (±S.E.M.) of data collected from three to five cells (*$P < 0.05$, *t*-test).

disodium (CNQX) suggesting that they are monosynaptic and mediated by glutamate (Bradley et al., 2000). Since there was no significant difference between results obtained with STN stimulation and results obtained with peduncle stimulation, data from the two sets of experiments were pooled in this study.

Activation of group I mGluR inhibits excitatory synaptic transmission in SNr neurons

All recordings were performed at a holding potential of −60 mV in the presence of bicuculline (10 μM) to block inhibitory synaptic transmission.

Brief bath application of the group I mGluR selective agonist DHPG (100 μM) produced a significant depression of EPSCs in GABAergic SNr neurons (Fig. 1A; $P < 0.05$; $n = 6$). This effect of DHPG was reversible (Fig. 1B). The concentration–response relationship for the DHPG-induced depression of EPSCs in SNr neurons revealed an EC_{50} around 5 μM with a maximal effect of 44.5 ± 2.5% at a concentration of 100 μM DHPG ($n = 6$, Fig. 1C). This is a concentration consistent with an effect on group I mGluRs (Schoepp et al., 1994).

Taken together, these data suggest that activation of group I mGluRs reduces excitatory synaptic transmission in the SNr. Since both group I mGluR subtypes, mGluR1 and mGluR5, are present in STN and SNr neurons (Testa et al., 1994), we used newly available pharmacological tools that distinguish between mGluR1 and mGluR5 to determine which receptor subtype mediates the depression of excitatory transmission in the SNr. Prior application (10–15 min) of MPEP (10 μM), a highly selective, non-competitive antagonist of mGluR5, showed no significant effect in blocking the effect of 50 μM DHPG on excitatory synaptic transmission in the SNr at concentrations shown to be effective at blocking mGluR5 effects in other systems (Gasparini et al., 1999; Bowes et al., 1999) and postsynaptic mGluR5 in both STN and SNr (Marino et al., 1999; Awad et al., 2000) (Fig. 2B,D). The mGluR1 selective, competitive antagonist LY367385 (300 μM) (Clark et al., 1997), in contrast, completely blocked the effect of 50 μM DHPG on excitatory synaptic transmission in the SNr (Fig. 2C,D). Taken together, these data suggest that activation of mGluR1 reduces glutamatergic synaptic transmission in the SNr.

The inhibitory effect of mGluR1 activation on synaptic transmission is mediated by a presynaptic mechanism

To test the hypothesis that this effect is mediated by presynaptic mechanisms we determined the effect of DHPG on paired-pulse facilitation of evoked EPSCs. All paired-pulse recordings were performed at a holding potential of −60 mV in the presence of bicuculline (10 μM) and EPSCs were evoked by stimulating the cerebral peduncle every 20 s by paired stimulations of equal strength at 20–100-ms intervals. Stimulus strength and inter-pulse intervals were adjusted in each experiment so that the second EPSC was always greater in amplitude than the first (paired-pulse facilitation: 139.8 ± 9.8%, $n = 5$). DHPG (100 μM) reduced the absolute amplitude

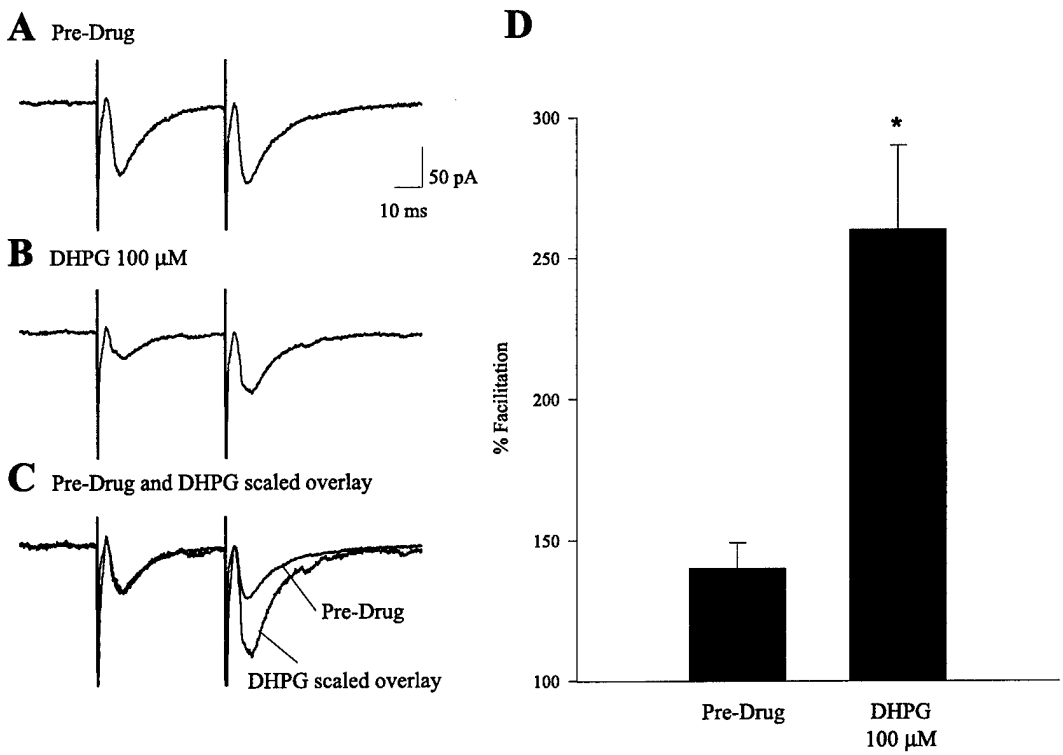

Fig. 3. Activation of the group I mGluR subtype, mGluR1, increases the ratio of paired-pulse facilitation of evoked EPSCs. (A, B) Representative traces of paired-pulse facilitation before (Pre-Drug) and during application of 100 μM DHPG. (C) Superimposed traces of pre-drug condition and during application of DHPG (trace scaled to first EPSC of control condition). (D) Bar graph showing the average effect of DHPG on the ratio of paired-pulse facilitation. Each bar represents the mean (±S.E.M.) collected from five cells (*$P < 0.01$; t-test).

of EPSCs but also increased the ratio of paired-pulse facilitation significantly to 260.4 ± 37.4% (Fig. 3, $P < 0.01$, $n = 5$, t-test). This represents an 88.9 ± 28.5% increase of facilitation induced by DHPG. Taken together, these data provide strong support for the hypothesis that DHPG acts presynaptically to inhibit the evoked release of transmitter from glutamatergic terminals.

Presynaptic localization of mGluR1a in the SNr

In order to confirm the presence of presynaptic mGluR1a in the SNr, we used a monoclonal antibody to immunohistochemically localize mGluR1a at the electron microscope level. Consistent with previous findings, dendritic elements of various sizes were strongly immunoreactive (Fig. 4A, B) (Hubert and Smith, 1999). In addition to postsynaptic elements, numerous unmyelinated axons and a few axon terminals forming asymmetric synapses were immunolabeled (Fig. 4A–C). While the identity of the small unmyelinated axons cannot be definitively determined, these structures are reminiscent of what would be expected for preterminal axonal segments. This, coupled with the presence of mGluR1a on terminals forming asymmetric synapses, suggests that mGluR1a is localized on presynaptic elements of excitatory synapses.

DISCUSSION

The data presented in this study reveal that activation of group I mGluRs reduces glutamatergic synaptic transmission in GABAergic SNr neurons and that this effect is mediated by a presynaptic mechanism.

We have previously shown that stimulation of the STN with the protocol used in this study induces glutamatergic EPSCs in SNr neurons (Bradley et al., 2000). In addition, since a large percentage of excitatory terminals on SNr GABAergic neurons arise from the STN (Smith et al., 1998), it is very likely that the observed inhibitory effect of DHPG on glutamatergic synaptic transmission is mediated by action on group I mGluRs localized at STN–SNr synapses. However, since the STN is not the only source of asymmetric synapses observed in the SNr, effects on other glutamatergic synapses cannot be excluded.

Our immunocytochemical studies suggest that the group I mGluR subtype mGluR1a is localized on unmyelinated axons in the SNr and is also found presynaptic in a few terminals forming asymmetric synapses. This is in agreement with our pharmacological studies showing that the inhibition of EPSCs induced by DHPG is solely mediated by the subtype mGluR1 but not mGluR5. Furthermore, our findings that DHPG

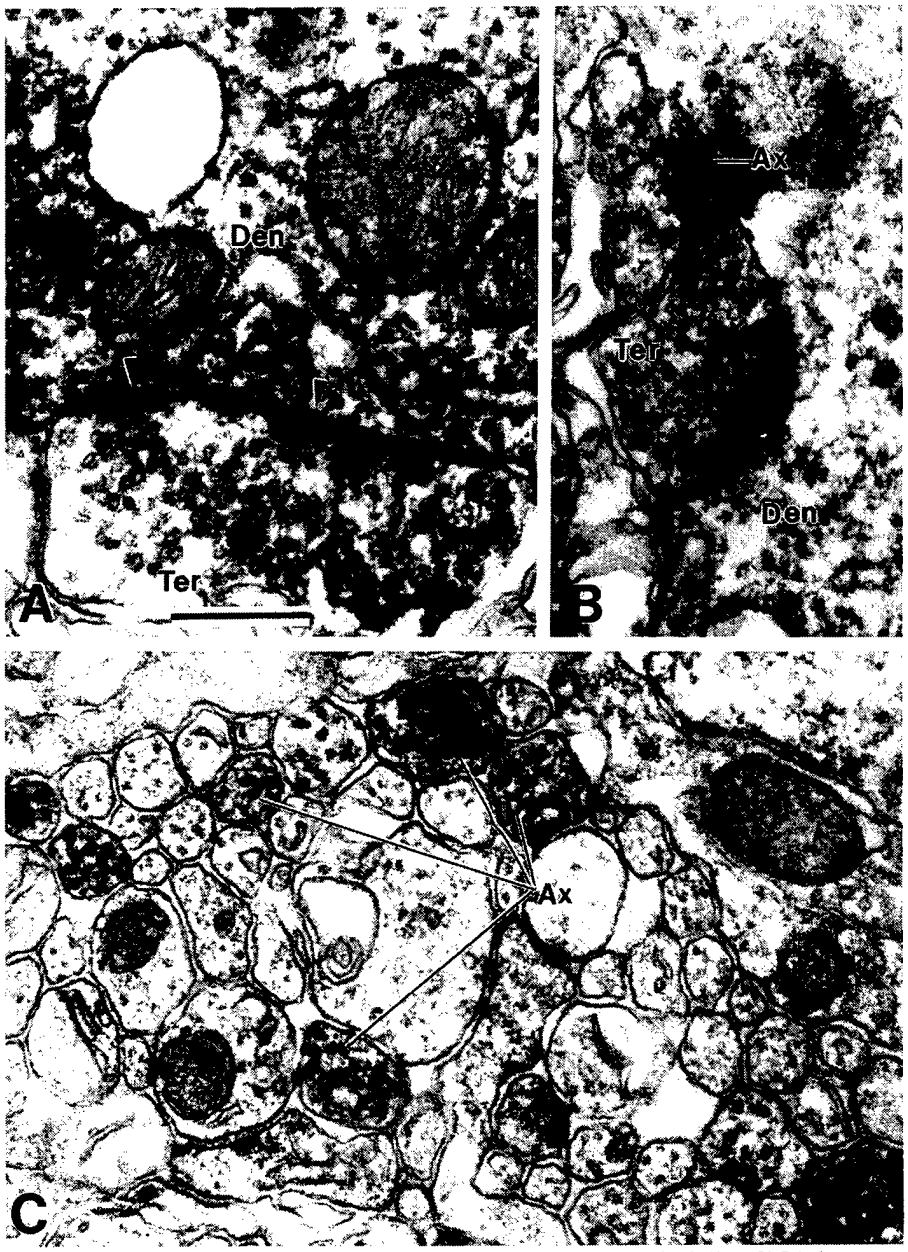

Fig. 4. Immunoperoxidase mGluR1a labeling in the rat SNr at the electron microscope level. (A) mGluR1a-immunoreactive terminal (Ter) forming an asymmetric synapse (arrowheads) with an immunoreactive dendrite (Den). (B) mGluR1a-immunoreactive terminal in the monkey SNr apposed to an mGluR1a-immunoreactive dendrite. Due to the dense peroxidase deposit, the synaptic specialization cannot be visualized. Note that the preterminal portion of the axon (Ax) is also strongly immunoreactive. (C) mGluR1a-immunoreactive unmyelinated axons in the SNr. Scale bar = 1.0 μm.

increases the ratio of paired-pulse facilitation indicates that this effect is mediated by a presynaptic mechanism.

Both group I mGluR subtypes, mGluR1 and mGluR5, are expressed in STN neurons (Awad et al., 2000). Despite the coexistence of the two mGluR subtypes, a recent *in vitro* study shows that DHPG-induced depolarization and potentiation of postsynaptic NMDA receptor currents in STN neurons are exclusively mediated by the mGluR5 subtype (Awad et al., 2000). This suggests that there might exist a difference in trafficking for the two group I mGluR subtypes in STN neurons. While the subtype mGluR5 is mainly localized postsynaptically in dendrites of STN neurons, the subtype mGluR1 could be trafficked to STN terminals, acting as a presynaptic

autoreceptor. Taken together, our data indicate that DHPG decreases synaptic transmission at STN–SNr synapses by activation of the presynaptically localized group I mGluR subtype mGluR1.

However, it should be noted that other mechanisms might mediate the effects of group I mGluR activation on EPSCs. For instance, studies in hippocampal CA1 pyramidal cells (Alger et al., 1996; Morishita et al., 1998; Morishita and Alger, 1999) and cerebellar Purkinje cells (Llano et al., 1991; Vincent et al., 1992; Vincent and Marty, 1993) suggest that depolarization-induced release of an unknown retrograde messenger can reduce inhibitory synaptic transmission by acting at presynaptic sites on GABAergic terminals. This so-called depolarization-induced suppression of inhibition involves a transient ($\sim$1 min) suppression of $GABA_A$ receptor-mediated inhibitory postsynaptic currents impinging on these cells after depolarization of their membranes that is sufficient to open voltage-gated Ca^{2+} channels. We have previously shown that activation of mGluR1 induces a robust depolarization of GABAergic SNr neurons by acting on postsynaptically localized receptors (Marino et al., 1999, 2000). This raises the possibility that the effect of DHPG on excitatory synaptic transmission might be induced by a postsynaptic mGluR1-mediated depolarization of SNr neurons and subsequent release of a retrograde messenger which, then, acts on presynaptic sites in glutamatergic axons and terminals.

Although the most common role of group I mGluRs is the postsynaptic regulation of neuronal excitability, activation of group I mGluRs has been shown to decrease glutamate release in other brain regions, including the CA1 region of the hippocampus (Gereau and Conn, 1995; Manzoni and Bockaert, 1995). Activation of presynaptic mGluRs can also facilitate glutamate release (Herrero et al., 1992) probably due to diacylglycerol production and protein kinase C activation (Herrero et al., 1994; Coffey et al., 1994). Interestingly, recent electrophysiological and biochemical studies in hippocampus and cerebral cortex suggest that presynaptically localized group I mGluRs undergo an activity-dependent switch where activation causes, first, a facilitation of synaptic transmission and then a depression (Herrero et al., 1998; Rodriguez-Moreno et al., 1998). It has been suggested that this is mediated by desensitization of signaling pathways involved in facilitation of release so that inhibition of release predominates after prolonged agonist application. We did not see evidence of this biphasic effect on EPSCs in the SNr. However, detection of the facilitatory phase in hippocampus requires relatively rapid agonist application that was not used in the present study. It is noteworthy that even the increased ambient concentration of extracellular glutamate due to slice preparation may induce the desensitization of the facilitatory response, switching the receptor function to inhibition in hippocampal slices (Rodriguez-Moreno et al., 1998). It is therefore possible that, in our slices, the extracellular glutamate concentration is high enough to act on presynaptic group I mGluRs thereby inducing a switch of the receptor state towards inhibition of glutamate release.

Our current findings add to a growing body of literature suggesting that group I mGluRs play an important role in regulating basal ganglia functions. Both group I mGluR subtypes are expressed throughout the basal ganglia (Testa et al., 1995; Kerner et al., 1997; Kosinski et al., 1998; Hanson and Smith, 1999; Smith et al., 2000) and have been shown to modulate neuronal activity in various basal ganglia structures. For instance, activation of group I mGluRs potentiates NMDA receptor currents in striatal medium spiny neurons (Colwell and Levine, 1994; Pisani et al., 1997). Furthermore, behavioral studies combined with measurements of changes in 2-deoxyglucose uptake and c-*fos* expression suggest that injection of group I mGluR agonists in the striatum selectively increases transmission through the indirect pathway (Kaatz and Albin, 1995; Kearney et al., 1997). Previous anatomical studies showed that neurons in the STN express both group I mGluR subtypes (Testa et al., 1994, 1998; Awad et al., 2000). In line with these findings, recent electrophysiological data demonstrated that activation of group I mGluRs induces a robust depolarization and potentiates NMDA receptor currents in STN neurons (Awad et al., 2000). Interestingly this effect is selectively mediated by activation of the group I mGluR subtype mGluR5. Group I mGluRs have also been shown to increase cell excitability and potentiate NMDA receptor currents in the rat SNr (Marino et al., 1999, 2000). In contrast to STN neurons this effect is solely mediated by activation of the subtype mGluR1. Additionally, group I mGluRs decrease inhibitory synaptic transmission in GABAergic SNr neurons (Wittmann et al., 2000). Our finding that activation of mGluR1 decreases glutamate release in the SNr seems to be opposing the effects of released glutamate on postsynaptic group I mGluRs. However, it is conceivable that these opposing effects of glutamate at pre- and postsynaptic sites act in concert to provide a filter mechanism that increases the signal to noise ratio of transmission at STN–SNr synapses. While postsynaptic activation produces an increase in excitability (direct depolarization) and sensitivity to glutamate (potentiation of NMDA receptor currents) (Marino et al., 2000), presynaptic activation reduces synaptic transmission, thereby ensuring that only strong signals elicit a significant postsynaptic response. Because of the postsynaptic actions, these stronger signals would, then, have a larger effect on activity of neurons in the SNr. Future studies at the circuit and systems level will be required to gain a complete understanding of the overall impact of group I mGluR activation on transmission through the basal ganglia circuits.

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